(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 March 2001 (01.03.2001)

PCT

(10) International Publication Number
WO 01/13967 A1

(51) International Patent Classification[7]: A61L 15/28, 26/00, C07K 14/78

(21) International Application Number: PCT/CA00/00953

(22) International Filing Date: 21 August 2000 (21.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/149,958 20 August 1999 (20.08.1999) US
60/182,412 14 February 2000 (14.02.2000) US

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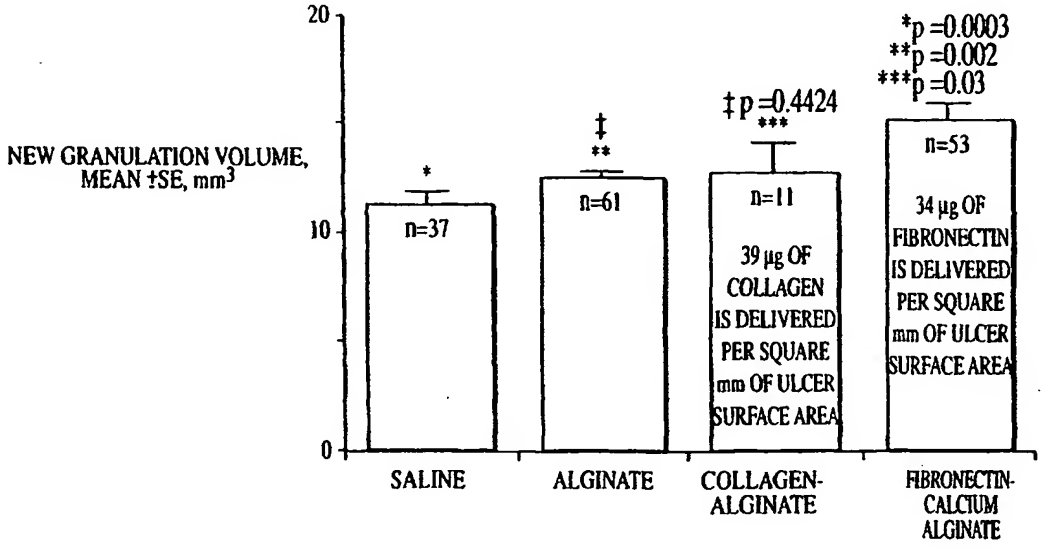
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SOLID WOUND HEALING FORMULATIONS CONTAINING FIBRONECTIN



(57) Abstract: A concentrated solution of a wound healing promoter, in particular fibronectin, is used to develop solid wound dressings. The solid wound dressings containing fibronectin are easy to apply, easy to remove, and deliver fibronectin in a range of physiologically active dosages.

WO 01/13967 A1

SOLID WOUND HEALING FORMULATIONS CONTAINING FIBRONECTIN

FIELD OF THE INVENTION

The present invention relates to solid wound dressings which release effective amounts of fibronectin, in particular fibronectin-calcium alginate dressings.

BACKGROUND OF THE INVENTION

Fibronectin is a ubiquitous extracellular glycoprotein containing around 5% carbohydrate. It exists in a soluble form in body fluids and in an insoluble form in the extracellular matrix. Fibronectin plays a major role in many important physiological processes, such as embryogenesis, hemostasis, thrombosis and wound healing (Potts, J.R. and Campbell, I.D. Current Opinion in Cell Biology 6:648-55, 1994). The characteristic form of plasma fibronectin is a disulfide-bonded dimer of 440,000 daltons, each subunit having a molecular weight of about 220,000 daltons. Plasma fibronectin is also known by various other names, including cold-insoluble globulin, antigelatin factor, cell attachment protein, cell spreading factor, and opsonic alpha 2-surface binding glycoprotein. These names reflect biological activities of fibronectin such as cell recruitment, opsonization of particulate debris, and promotion of wound contraction. Reviews on structure and activities of fibronectin have been published elsewhere (Hynes, R.O. Fibronectins, Rich, A., ed. New York, Springer-Verlag 1990).

Wound healing is usually divided into three phases: the inflammatory phase, the proliferative phase, and the remodeling phase. Fibronectin has been reported to be involved in each stage of the wound healing process, particularly by creating a scaffold to which the invading cells can adhere. Initially, there is a release of many mediators to the wound site such as fibronectin and fibrinogen.

Fibronectin promotes inflammatory cell migration into the wound and debris phagocytosis by monocytes. Thereafter, angiogenesis and reepithelialization take place. At this stage, fibronectin exerts chemotactic activity on endothelial cells, and promotes epithelial cell and fibroblast migration onto the basal membrane. Fibronectin also appears to be an essential component of the remodeling phase where it plays a major role in the organization of collagen fibrils. The fibrillar collagen ultimately forms fibrous bundles that greatly enhance the tissue tensile strength, leading to wound closure. Normally found in plasma at a concentration of about 300 µg/mL, fibronectin is extracted and purified using a method developed by Horowitz and Chang (Horowitz, B. and Chang, M.D.Y. "Preparation of fibronectin for therapeutic administration in Fibronectin, D.F. Mosher ed., Academic Press, San Diego 441-455 (1989)).

Topically applied plasma fibronectin has been reported as being useful for increasing the rate of wound healing such as in corneal wounds (Nishida, T. et al., Japan Journal of Ophthalmology 26: 416-24, 1982; Phan, T.M. et al., American Journal of Ophthalmology 104:494-501, 1987) and leg ulcers (Wysocki, A. et al., Arch. Dermatology 124: 175-177, 1988). However, there is no suitable topical carrier for use in treating wounds that can ensure delivery of an effective amount of fibronectin in a pharmaceutically acceptable formulation. A major limiting factor in developing an effective topical dosage form of a drug is not only having an active drug, but also having a formulation that allows the passage of the active drug from the carrier into a site of delivery.

A topical formulation, which maximizes the contact time of fibronectin to the wound and controls the release of fibronectin into the wound, is a hydrogel formulation. In drug delivery, the term hydrogel is typically reserved for polymeric materials that can absorb a significant amount of water (> 20% of its dry weight) while

maintaining a distinct three-dimensional structure (Gehrke, S.H. and Lee, P.I., "Hydrogels for drug delivery systems," *In* Specialized Drug Delivery Systems Manufacturing and Production Technology, Chapter 8, Vol. 8, PP 333-392, Marcel Dekker, New York 1990). The most important characteristic of a hydrogel is its degree of swelling in water. Hydrogels mimic living tissue more closely than any other non-natural material. Their immediate resemblance to tissue is in their soft, flexible nature and high water content. This helps minimize mechanical irritation and damage to body tissues. Other advantages of hydrogel formulations include: ability to keep the wound moist which results from their high water content, ability to absorb excess water (exudate) in the wound, ease of application to and removal (by washing) from the wound. They also provide a cool feeling when topically applied, a property that can increase patient comfort.

Hydrogels have four major properties: swelling degree, biocompatibility, permeability and swelling kinetics. Example of such compounds include vinyl polymers (e.g. polyacrylic acid), cellulose and cellulose derivatives. Polyacrylic acid polymer, also referred to as carbomer, e.g. Carbopol® carbomer (BF Goodrich), was chosen over other polymers (e.g. cellulose and cellulose derivatives), because it was shown to be superior to other pharmaceutically acceptable formulations in the delivery of fibronectin to skin wounds.

Hydrogel formulations comprising a water soluble, pharmaceutically acceptable polymer which can include increasing concentrations of fibronectin are described in U.S. Patent 5,641,483, entitled "Wound Healing Formulations containing Human Plasma Fibronectin", which is incorporated by reference herein in its entirety. Methods for preparing non-buffered aqueous concentrated solutions of fibronectin and hydrogels containing up to 1% of fibronectin are described in U.S. Patent No. 5,821,220, entitled "Method of Producing Concentrated Non-Buffered Solutions of Fibronectin" and

International Application No. PCT/CA97/00966, International Publication No. WO 98/26797, entitled "Wound Healing Formulations Containing Human Plasma Fibronectin", both of which are incorporated herein by reference in their entirety.

Alginates are naturally occurring substances extracted from marine brown algae and used in the pharmaceutical, cosmetic, textile and food industry. Alginates are polyanionic polysaccharides composed of linear binary copolymers of D-mannuronic acid and L-guluronic acid. The most common uses are based on the polyelectrolytic nature of the alginates, which provides the basis of their gelling properties and their ability to swell. The commercially available sodium alginates are water soluble. When such alginates are added to a solution containing polyvalent ions, for example bivalent alkaline earth metal ions such as Ca^{++}, alginate gels having a semi-solid form are produced. This is a result of a ionic crosslinking of several alginate chains.

Calcium alginates have long been known for their ability to form fibres or nonwoven materials. These have been used primarily as swabs or dressings for medical, surgical or other purposes, such as described in European Patent Specification, EP 0721355 B1, entitled "Alginate Wound Dressings, which is incorporated herein by reference in its entirety. Supplied in the form of nonwoven wound dressings for the treatment of exudating wounds, the calcium alginate dressing is said to encourage the formation of controlled ion-active gel over the wound site which reacts with the sodium ions in the exudate. Examples of exudative wounds include pressure ulcers, venous stasis ulcers, diabetic ulcers, arterial ulcers, second degree burns and skin graft donor sites.

SUMMARY OF THE INVENTION

The present invention provides techniques for the creation of solid wound dressings capable of delivering an effective wound healing amount of fibronectin to a wound site. Examples of solid dressings used to deliver the fibronectin are based on calcium alginate, carboxymethylcellulose (CMC), hydroxypropylcellulose (HPC), carbomer and carrageenan.

Formulation of topical dosage forms intended for the incorporation of fibronectin should respect several quality criteria. All components of the preparation including solvents and gelling agents should be nontoxic for the wound and compatible with the drug. The final product should promote optimal release of the drug to its site of action, be of adequate consistency to enhance contact time of the drug with the wound and be sterile.

Use of solid dressings of the present invention offer specific advantages in terms of dose reproducibility, ease of storage, transport and application. In addition, preservatives are not needed.

These solid dressings provide a slow release of fibronectin to the delivery site. This should allow for the application of the solid wound dressings on a convenient once a day basis. Due to once a day application schedule and their solid form, the trauma done to the wound by the removal of depleted doses should be minimized.

The preferred formulations of this invention can be used with other wound healing promoters having a composition similar to fibronectin, such as proteins of similar size (thrombospondin, laminin, vitronectin, fibrinogen) or smaller size (such as peptides including growth factors).

The preferred formulations can be evaluated using an *in vitro* diffusion cell system consisting of a rigid receptor containing a deepithelialized skin sample, the deepithelialized side facing upwards into a donor compartment and the dermal side facing downwards into a receptor compartment as described in detail in U. S. Patent No.

5,877,149, entitled "Deepithelialized Skin Diffusion Cell System," which is incorporated herein by reference. The receptor compartment is connected to a circulating buffer circuit, with the buffer temperature maintained at 37°C, while the skin surface is about 32°C.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows absorption of fibronectin in deepithelialized human skin using different solid wound dressings over a 24 hour period. The number in ( ) refers to the quantity of absorbed fibronectin (μg) per mm^2 of deepithelialized human skin over a 24 hour period. The number in [ ] refers to the quantity of absorbed saline solution (0.9% NaCl) by weight of dressing. Bars represent standard deviations of the mean.

Figure 2 depicts new granulation tissue formation, measured as maximum height (μm) in response to treatment with a fibronectin-calcium alginate dressing after 7 days of treatment. 6-mm diameter dressings were applied at the time of surgery. Alginate dressing (Kaltostat), collagen-alginate dressing (Fibracol) and fibronectin-calcium alginate dressings were placed on the ulcers and wetted with 40 μL of saline solution. Control treatment (identified as †"saline") were ulcers treated with 40 μL of saline alone. Occlusive dressings (Tegaderm) were used to prevent wound desiccation. The fibronectin-calcium alginate dressing (n=53) was significantly better than saline ($P < 0.0001$, n=37), alginate ($P < 0.0001$, n=61) or collagen-alginate ($P < 0.0001$, n=11). No difference was observed between alginate and collagen-alginate dressing ($P = 0.1052$) using Student two-tailed *t* test. Bars represent standard errors of the mean.

Figure 3 illustrates the new granulation volume formed in response to treatment with fibronectin-calcium alginate dressing after

7 days of treatment. 6-mm diameter dressings were applied at the time of surgery. Alginate (Kaltostat), collagen-alginate (Fibracol) and fibronectin-calcium alginate dressings were placed on the ulcers and wetted with 40 µL of saline solution. Control treatment (identified as †"saline") were ulcers treated with 40 µL of saline alone. Occlusive dressings (Tegaderm) were used to prevent wound desiccation. The fibronectin-calcium alginate dressing (n=53) was significantly better compared to saline ($P < 0.0003$, n=37), alginate ($P < 0.002$, n=61) or collagen-alginate ($P < 0.03$, n=11) dressing in stimulating new granulation tissue formation. No difference was observed between alginate and collagen-alginate dressing ($P=0.4424$) using Student two-tailed *t* test. Bars represent standard errors of the mean.

Figure 4 shows new granulation tissue formation, measured as maximum height (µm) in response to 1.0 % fibronectin carbomer hydrogel after 7 days of treatment. The fibronectin was applied as a 0.281% carbomer hydrogel (40µL containing 400 µg of fibronectin) at the time of the surgery and daily during 7 days. The volume of the topical formulations applied to control wounds (saline and 0.281% carbomer hydrogel) was also 40 µL. Antiseptic tulle gras dressing (Bactigras) and non-adherent absorbent dressing (Melolite) were used to prevent wound desiccation. The 1.0% fibronectin-carbomer hydrogel treatment (n =45) was significantly better than either saline ($P = 0.01$, n=43) or carbomer hydrogel containing no fibronectin in stimulating new granulation tissue formation ($P = 0.001$, n=43). No difference was observed between saline and carbomer hydrogel alone ($P = 0.1752$) using Student two-tailed *t* test. Bars represent standard errors of the mean.

Figure 5 illustrates the new granulation volume in response to 1.0% fibronectin carbomer hydrogel after 7 days of treatment. The

fibronectin was applied as a 0.281% carbomer hydrogel (40µL containing 400 µg of fibronectin) at the time of the surgery and daily during 7 days. The volume of the topical formulation applied to the control wounds (saline and 0.281% carbomer hydrogel) was also 40 µL. Antiseptic tulle gras dressing (Bactigras) and non-adherent absorbent dressing (Melolite) were used to prevent wound desiccation. The 1.0 % fibronectin-carbomer hydrogel treatment (n=45) was significantly better when compared to saline (P = 0.0285, n=43) and carbomer hydrogel containing no fibronectin (P =0.0238, n=43) in stimulating new granulation tissue formation. No difference was observed between saline and carbomer hydrogel alone (P =0.4152) using Student two-tailed t test. Bars represent standard errors of the mean.

Figure 6 illustrates the amount of fibronectin absorbed from fibronectin-calcium alginate disks prepared according to Example 1 for various tested amounts of fibronectin. Absorption was measured using the human deepithelialized skin diffusion cell system after 24 hours.

DETAILED DESCRIPTION OF THE INVENTION

Example 1

Solid fibronectin calcium-alginate wound dressing formulation

Alginate salts, as well as the dressing systems described in subsequent examples, can be converted into fibers by a process of freeze-drying. This procedure produces a sponge-like structures with hydrophilic properties. In the presence of fluids, the dressings turn into a gel-like state, capable of absorbing up to 20 times their weight in wound exudate. The fibrous gel thereby creates the desired moist environment for the wound. The dressings can also be removed with a minimal amount of discomfort, minimizes the formation of granulation

tissue and does not traumatize epithelial cells during dressing changes.

The fibronectin-calcium alginate and other fibronectin solid wound dressings according to the invention can store fibronectin without degradation for long periods of time, at least 12 months at 4°C. The residual moisture in these dressing is low, around 5%.

The fibronectin-calcium alginate and other fibronectin-solid wound dressings according to the invention deliver a high concentration of fibronectin into the wound site.

The basic mechanisms at play for the fibronectin-calcium alginate dressing is that when this dressing comes into contact with the sodium in the exudate, ion exchange occurs, turning the calcium alginate fibers into a protective non-adherent film gel. In this gel state, fibronectin is free to move from the gel into the wound.

Solubilization

$$Ca\ (alginate)_2 + 2\ Na^{+} \text{ ----------> } 2\ Na\ (alginate) + Ca^{++}$$

Ca^{++} forms an insoluble alginate salt and Na^{+} forms a soluble alginate salt (the equivalent ratio of the first to second cations being 50:50, here 0.2M NaCl and 0.2M $CaCl_2$). The maximum homogeneity in the dressing is reached by an appropriate concentration of both gelling and non-gelling cations. Additional Na+ comes from the exudate or, if the wound is too dry or there is no exudate, a small amount of saline can be added to the wound immediately prior to placing the dressing.

Because alginates are anionic polysaccharides, the complex is preferably formed by combining the fibronectin and sodium alginate at a pH which is no higher than the isoelectric point of the protein (pI 6.2) where the fibronectin is positively charged. This pH is achieved

by adding glacial acetic acid for a final pH around 5.0. Since acetic acid is highly volatile, a certain amount of acetic acid is removed during the freeze-drying process which is under a vacuum. The final pH of the dressing is around 6.5.

The mixed salt alginate dressing exhibits a highly effective combination of properties. For example, there is enough insolubilizing cation in the mixed salt alginate to make the product relatively easy to manipulate. There is also enough solubilizing cation to facilitate the release of fibronectin into the wound. The combination of soluble and insoluble alginate fibers has the further advantage that the dressing is both easily removed after the wound treatment and easily applied initially.

Fibronectin-calcium alginate dressing composition

Three commercially available preparations of sodium alginate were tested. Protanal LF 120 M sodium alginate (Pronova Biopolymer, Inc., Drammen, Norway) yielded a product more brittle than the preferred embodiment described below. In addition, this sodium alginate yielded a placebo dressing (i.e., control without fibronectin) which was yellowish in appearance compared to the same dressing containing fibronectin. Consequently, this formulation could not be used in human clinical trials. A particularly preferred alginate is Pronova UP LVG sodium alginate (Pronova Biomedical A.S., Oslo, Norway).

In one embodiment of the present invention, the fibronectin-calcium alginate wound dressing is prepared as follows: 5 g of sodium alginate (Protanal LF 10/60, Pronova Biopolymer, Drammen, Norway) are dispersed in 95 g of deionized and demineralized water with a paddle type stirrer for about 1 hour. The colloidal dispersion thus produced provides a concentrated sodium alginate base (5 % w/w),

which is then autoclaved. The pH of a sterile filtered 1% solution of sodium alginate prepared from this concentrate is adjusted to 4.0 using acetic acid (3.33 µL of glacial acetic 17.4N in 100 Ml of demineralized water provides a pH of 4.0). Tap water is demineralized using a Millipore Milli-Q water system. The terms demineralized and deionized are used interchangeably throughout this application. The pH of 10 mL demineralized water is adjusted to pH 8.0 to 11.0 as described in Example 9. In the following preferred embodiment, the pH is adjusted to 11.6.

0.025 to 0.1 g, preferably 0.1 g, of lyophilized human plasma fibronectin, prepared according to Example 10, is next dissolved in demineralized water, pH 11.6. The solution is maintained at 37°C until the fibronectin is completely dissolved. The fibronectin solution is then filtered through a 0.22 µm acetate filter. This solution constitutes the stock fibronectin solution used in this and subsequent examples.

3.4 ml of the resulting 0.25% to 1% sterile solution of fibronectin is then mixed with 1.5 mL of the pH adjusted, dilute sodium alginate solution described above. The sterile fibronectin and sterile sodium alginate solutions are mixed into syringes taking care to avoid the introduction of air bubbles. Contamination is avoided by working in an aseptic environment, such as under a laminar flow hood. Generally, two syringes are used, and multiple exchanges under pressure are applied. An adapter device, such as a female luer connection can be used to connect the syringes or other exchange devices. Vigorous agitation is minimized in order to avoid fibronectin precipitation.

Gellation of the solution is achieved by the addition of 15 µL 0.2 M NaCl + 0.2 M $CaCl_2$ and 3.4 µL of glacial acetic acid. At this point, the fibronectin-calcium alginate complex is deposited in a borosilicate glass vial (5 mL for a surface area of 5.3 cm^2) and frozen at -20°C for

2 hours and 30 minutes at -80°C. A preferred vial is 22.5 mm in width, 46.5 mm in height with an aluminum seal (part no. 24-0396, Comar, Buena, NJ). The water is then removed by freeze-drying using a Labconco freeze-dryer (model 77580, Kansas City, MO). By this technique, a solid sponge-like fibronectin-calcium alginate wound dressing is produced, which has a surface area of 4.2 cm^2 and a fibronectin concentration up to 80 $\mu g/mm^2$. It should be noted that solid wound dressings could also be prepared with other insoluble fibers. They could be any insoluble fibers or materials which does not have adverse effect on the wound. Examples of suitable plant polysaccharides are carrageenans and cellulose derivatives for instance carboxymethylcellulose or hydroxypropylcellulose are described in the following examples. An embodiment using a synthetic carbomer resin is also illustrated. Tissue matrices and artificial skin systems, for example, as described in U.S. Patent 4,963,489, entitled "Three-Dimensional Cell and Tissue Culture system, incorporated by reference, can also be employed in embodiments of the present invention.

Example 2

Solid Carboxymethylcellulose (CMC) dressing

A preferred grade is GPR® CMC (BDH Laboratories, Ville St-Laurent, Canada). A solid wound dressing containing (w/w) fibronectin 62%, CMC 38% was prepared as follows. CMC powder was first sterilized by using a dry-heat sterilization process at 121°C for 30 minutes using an American Sterilizer 2020 Vacamtic Eagle series autoclave (Steris Corp., Ohio). 6 grams of CMC were dispersed in 94 mL of demineralized water and mixed with a paddle type stirrer for about 3 hours. This provides a sterile concentrated hydrogel base (6% w/w).

Separately, 50 mg of lyophilized human plasma fibronectin, prepared according to Example 10, was dissolved in deionized water (5

mL) containing 12 µL of NaOH 3M, for a final pH of 11.6. The solution was maintained at 37°C until the complete solubilization of fibronectin occurred. This stock solution of fibronectin 10 mg/mL was filtered through a 0.22 µm acetate filter. 3.3 mL of this fibronectin solution was then added to 0.34 g of the concentrated CMC base and mixed with syringes as described in Example 1. The pH is adjusted to 7.0 with the addition of 25 µL HCl 1N. At this point, the homogenous solution of the fibronectin-CMC complex was deposited in a plastic mold and frozen. A Costar 6-well polystyrene cell culture cluster, 9.6 cm^2 rounded surface area (Corning Inc., Corning, NY) was used as the plastic mold. The water is then removed by freeze-drying using a Labconco freeze-dryer (model 77580, Kansas City, MO). By this technique, a fibronectin-CMC wound dressing with a sponge-like structure is produced.

Example 3

Solid Hydroxypropylcellulose (HPC) dressing

Solid hydroxypropylcellulose (HPC) dressing was prepared using the preferred grade of Klucel-HF® HPC (Aqualon, Houston, Texas). A solid wound dressing containing (w/w) fibronectin 45%, HPC 55% was prepared as follows. HPC powder was first sterilized by using a dry-heat sterilization process at 121°C for 30 minutes using an American Sterilizer 2020 Vacamtic Eagle series autoclave (Steris Corp., Ohio). HPC (6 g) was then dispersed in 94 mL of deionized water and mixed with a paddle type stirrer for about 3 hours. This provides a sterile, concentrated hydrogel base (6% w/w). Separately, 50 mg of lyophilized human plasma fibronectin, prepared according to Example 10, were dissolved in 5 mL of deionized water containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C until complete solubilization of fibronectin occurred. This stock solution of

fibronectin, 10 mg/mL, was filtered through a 0.22 µm acetate filter. 3.3 mL of the fibronectin solution was then added to 0.68 g of concentrated HPC base and mixed with syringes as described in Example 1. The pH is adjusted to 7.0 with the addition of 25 µL HCl 1 N. At this point, the homogenous solution of the fibronectin-HPC complex is deposited in a plastic mold and frozen. A Costar 6-well polystyrene cell culture cluster, 9.6 cm^2 rounded surface area (Corning Inc., Corning, NY) was used as the plastic mold. The water is then removed by freeze-drying using a Labconco freeze-dryer (model 77580, Kansas City, MO). By this technique, a fibronectin-HPC wound dressing with a sponge-like structure is produced.

Example 4

Solid carbomer dressing

A solid carbomer dressing was prepared using Carbopol® 974P NF carbomer (BF Goodrich, Cleveland, Ohio) as the preferred grade. A solid wound dressing containing (w/w) fibronectin 75%, carbomer 25% was prepared as follows. 2.80 g of carbomer was dispersed in 97.2 mL of demineralized water and mixed with a paddle type stirrer for about 3 hours. This dispersion is then autoclaved to provide a sterile concentrated hydrogel base (2.80% w/w). 50 mg of lyophilized human plasma fibronectin, prepared according to Example 10, were dissolved in 5mL of deionized water containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C until complete solubilization of fibronectin occurred. This stock solution of fibronectin (10 mg/mL) was filtered through a 0.22 µm acetate filter. 3.3 mL of the fibronectin solution was then added to 0.04g of concentrated carbomer base and the necessary amount of gelifying promoter (25 µL NaOH 3M) and mixed with syringes as described in Example 1. This fibronectin carbomer hydrogel is deposited in a plastic mold and frozen. A Costar 6-well polystyrene cell culture

cluster, 9.6 cm^2 rounded surface area (Corning Inc., Corning, NY) was used as the plastic mold. The water is then removed by freeze-drying using a Labconco freeze-dryer (model 77580, Kansas City, MO). By this technique, a fibronectin-carbomer wound dressing with a sponge-like structure is produced.

Example 5

Solid carrageenan dressing

Solid carrageenan dressing was prepared. The preferred grade is Gelcarin® NF carrageenan (FMC Corporation Pharmaceutical Division, Newark, Delaware). A solid wound dressing containing (w/w) fibronectin 73%, carbomer 27% was prepared as follows. 2.50 g of carrageenan was dispersed in 97.5 mL of deionized water and allowed to be mixed with a paddle type stirrer for about 3 hours. This dispersion is then autoclaved to provide a sterile concentrated hydrogel base (2.50% w/w). 50 mg of lyophilized human plasma fibronectin, prepared according to Example 10, were dissolved in 5 mL of deionized water containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C until complete solubilization of fibronectin occurred. This stock solution of fibronectin (10 mg/mL) was filtered through a 0.22 µm acetate filter. 3.3 mL of fibronectin solution was then added to 0.50 g of concentrated carrageenan base and mixed with syringes as described in Example 1. The pH is adjusted to 7.0 with the addition of 60 µL HCl 1 N. At this point, the homogenous solution of the fibronectin-carrageenan complex is deposited in a plastic mold and frozen. A Costar 6-well polystyrene cell culture cluster, 9.6 cm^2 rounded surface area (Corning Inc., Corning, NY) was used as the plastic mold. The water is then removed by freeze-drying using a Labconco freeze-dryer (model 77580, Kansas City, MO). By this technique, a fibronectin-carrageenan wound dressing with a sponge-like structure is produced.

Example 6

In vitro study of solid wound dressings

In vitro study of the absorption of fibronectin in deepithelialized human skin using a cell diffusion system showed that up to 91.7% of the fibronectin is released within a 12-hour period (see Fig. 1) from a fibronectin-calcium alginate dressing prepared as follows: 10.0 gram of sodium alginate (Protanal LF 10/60, Pronova Biopolymer, Drammen, Norway) is dispersed in 90 g of deionized and demineralized water with a paddle type stirrer for about one hour. This dispersion is then autoclaved to provide a sterile concentrated alginate base (10% w/w). 10 mL of a 1.0% fibronectin stock solution is filtered through a 0.22 μm acetate filter and mixed into syringes with 5 mL of a 1% sodium alginate solution in mild acetic acid, prepared from a concentrated alginate base. The gellation of the solution is achieved by adding 375 μL of (0.2M NaCl + 0.2 M $CaCl_2$) plus 30 μL of 17.45 N acetic acid for a final pH of 4.0. At this point, the homogenous solution of the fibronectin-calcium alginate complex is deposited in a plastic mold and frozen. A Costar 6-well polystyrene cell culture cluster, 9.6 cm^2 rounded surface area (Corning Inc., Corning, NY) was used as the plastic mold. The water is then removed by freeze-drying using a Labconco freeze-dryer (model 77580, Kansas City, MO). By this technique, a fibronectin-calcium alginate wound dressing with a sponge-like structure is produced. After freeze-drying, the surface area of the final disk is close to 8.96 cm^2 surface area because the disk shrinks during freeze-drying. The amount of fibronectin in the 5mL formulation is 33.3 mg for a concentration of 37 $\mu g/mm^2$ of surface area (33,333 μg/896 mm^2).

This fibronectin-calcium alginate dressing was compared to the solid wound dressings dressing of Examples 2-5 for 12 hours in deepithelialized skin diffusion cell system. The deepithelialized skin diffusion cell system utilized in the experiments shown in Figure 1 is

described in Beaulieu, U. S. Patent No. 5,877,149, issued March 2, 1999, which is incorporated herein by reference.

The amount released represents 34.1 μg of fibronectin per mm^2 deepithelialized skin surface area and is a significant increase (262%) compared with the 13.0 μg fibronectin released from 1.0% fibronectin carbomer hydrogel after 12 hours, as described in U.S. Patent No. 5,821,220 incorporated herein by reference. The amount of fibronectin released from the fibronectin-cellulose derivatives of Example 2 and 3 were similar with those obtained with fibronectin-calcium alginate (see Figure 1). However, once hydrated with saline solution, the fibronectin-cellulose dressings do not provide the desired fibrous protective film on the surface of the deepithelialized human skin.

Example 7

The rabbit ear dermal ulcer model

A study of the efficacy of the fibronectin-calcium alginate wound dressing in stimulating wound healing was performed using the rabbit ear dermal ulcer model of wound healing as developed by Mustoe et al. (1991) J. Clin Invest 87: 694-703.

Young adult New Zealand white rabbits, 3.0-3.5 kg (Charles River Laboratories, Canada) were anesthetized with ketamine (60 mg/kg) and xylazine (95 mg/kg). Using a 6-mm trephine and microsurgical instruments, four circular full-thickness 6-mm diameter ulcers were made to the depth of bare cartilage under sterile conditions. As cartilage is non-vascularized, new granulation tissue formation occurs only at the periphery of the ulcer.

6mm-diameter pieces of solid dressings were applied immediately after surgery. Alginate dressing (Kaltostat, ConvaTec,

Skillman, NJ), collagen-alginate dressing (Fibracol, Johnson & Johnson, Arlington, TX) and fibronectin-calcium alginate dressing were applied on the ulcers and wetted with 40 µL of saline solution (Aqualite® 0.9% sodium chloride solution). The fibronectin- calcium alginate dressing was prepared according to the method described in Example 1. Control treatment (identified in Figures 2-5) were ulcers treated with 40 µL of saline alone. The wounds were covered with an occlusive polyurethane film (Tegaderm film, 3M, Minneapolis, MN) to prevent wound desiccation. Neck collars were placed on rabbits for the duration of the experiment. Differences in rates of healing between treatment groups were measured at day 7.

A. Histomorphometric measurement of new granulation tissue

At the time of killing, the ulcers were bisected and fixed in 10% buffered formalin. The specimens were then dehydrated in graded alcohol and xylene, embedded in paraffin, and sectioned, taking care to obtain a cross section as near as possible to the center of the wound. After Masson-trichrome staining of 3-µm sections, the granulation tissue gap (GAP) (defined as the remaining diameter of the wound without new granulation tissue) and the maximum height (MH) of the new granulation tissue at the advancing edges of the wound were measured by histomorphometry using Biometrics Bioquant true color laser vision (R&M, Nashville, TN). Each MH value represents the mean of four measurements of the maximum height of the new granulation tissue for the right and the left side of two tissue sections for each ulcer.

The GAP distance is used to calculate the surface area of the wound by the equation $(GAP/2)^2\pi$. On day 0 (day of surgery), the measured GAP was 6.2 mm and the area was $(6.2/2)^2\pi = 30.19$ mm^2. New granulation tissue surface area is the area of wound at day 0 minus

the area of wound at day 7, i.e., $(GAP\ [day\ 0]/2)^2\pi - (GAP\ [day\ 7]/2)^2\pi$ The new granulation volume (NGV) is new tissue surface area x MH. Area and volume measurements for new granulation tissue were calculated based on the assumptions that the wounds healed concentrically and did not contract. Statistical analysis was carried out using a Student's paired *t* test for each formulations studied using Excel version 5.0 (Microsoft Corporation). All comparisons were made to paired control wounds (saline, alginate or collagen-alginate). $P < 0.05$ was considered significant.

B. Effect of fibronectin-calcium alginate dressing containing 65 % fibronectin (w/w) in stimulating new granulation tissue formation

Results of histomorphometric measurements show that the effect of a single application of the fibronectin-calcium alginate dressing, produced according to the method of Example 6, was significantly better than alginate (Kaltostat) or collagen-alginate (Fibracol) dressings in generating new granulation tissue in the rabbit ear dermal ulcer model. This amount of fibronectin delivered represents approximately 34 µg per mm^2 rabbit surface ulcer area whereas a dose of 39 µg per mm^2 of collagen was used for collagen-alginate dressing. As shown in Figure 2, after 7 days of treatment, the maximum height (MH) for fibronectin-calcium alginate treated wounds was significantly higher compared to the control wounds (732 ± 13 µm vs 599 ± 16 for saline, 648 ± 11 for alginate and 621 ± 27 for collagen-alginate treated wounds, $P < 0.0001$ in all cases). For the new granulation volume (NGV) values, significant increases (more than 120% compared to collagen-alginate, 122% compared to alginate and 137% compared to saline) were observed for the fibronectin-calcium

alginate (Figure 3). The calculated values were respectively 15.02 ± 0.77 mm^3 for fibronectin-calcium alginate, 10.95 ± 0.76 for saline, 12.29 ± 0.59 for alginate and 12.46 ± 1.47 collagen-alginate. Neither alginate nor collagen-alginate dressings were significantly different for the MH values ($P = 0.1052$) and NGV values ($P = 0.4424$) when compared to each other or saline.

C. Effect of 1.0% fibronectin-carbomer hydrogel

Treatment with fibronectin in a 1.0% fibronectin-carbomer hydrogel formulation on the rabbit dermal ulcer model was evaluated in its capacity to stimulate the formation of new granulation tissue. The fibronectin was applied as a 0.281% carbomer hydrogel, applying a volume of 40µL of hydrogel containing 400 µg of fibronectin. The1.0% fibronectin-carbomer hydrogel prepared according to Example 9, pH 11.6. This delivered approximately 13 µg of fibronectin per mm^2 rabbit ulcer surface area as described in U.S. Patent No. 5,821,220. The fibronectin-carbomer hydrogel was applied to the test ulcer once a day for a period of 7 days, starting at the time of surgery. The volume of topical formulations applied to the control wounds (saline or 0.281% carbomer hydrogel containing no fibronectin) was also 40 µL. Antiseptic tulle gras dressing (Bactigras®, Smith & Nephew, Lachine, Canada) and a non-adherent absorbent dressing (Melolite®, Smith & Nephew, Lachine, Canada) were used to prevent wound desiccation. Before each daily application, the ulcers were washed with sterile saline solution, gently cleaned with a moistened cotton-swab.

When the histomorphometric measurements were analyzed, they revealed that the mean maximum height of new granulation tissue in fibronectin-carbomer hydrogel-treated wounds (900 ± 30 µm, n=45) was significantly higher when compared to treatment with either saline (819 ± 23 µm, n=43, $P = 0.01$) or carbomer hydrogel

containing no fibronectin (793 ± 19 μm, n=43, P = 0.001), (Figure 4). Furthermore, the volume of new granulation tissue formation was significantly greater with 1.0% fibronectin-carbomer hydrogel treatment (21.22 ± 1.14 mm^3, n=45) when compared to saline (18.67 ± 0.79 mm^3, n=43, P = 0.0285) or carbomer hydrogel containing no fibronectin (18.43 ± 0.95 mm^3, n=43, P = 0.0238, (Figure 5). No difference was observed between saline and carbomer hydrogel alone. (P = 0.4152)

D. Dosage Testing of Fibronectin

A study of the effects of varying doses of fibronectin on wound healing was conducted in a rabbit model using techniques as described above. However, in these experiments, a 9-mm AcuPunch® biopsy punch was used to produce the model ulcers in the rabbit ear. Four wounds were made in each ear of each experimental animal. One ear was treated with the saline control dressing and the other ear was treated with the fibronectin calcium-alginate dressing as described above. The fibronectin-calcium alginate disks for this experiment are prepared as in Example 1. The disks were stacked and if necessary cut to provide the following dosages of fibronectin, 10.3 μg/mm^2, 20.6 μg/mm^2, 41.25 μg/mm^2, 82.5 μg/mm^2, 123.75 μg/mm^2, 165 μg/mm^2 . The results of varying dosages on maximum height (MH) and new granulation volume (NGV) are shown respectively, in Tables 1 and 2 below. Clear benefit from the presence of fibronectin-calcium alginate disks was demonstrated and the effect appears to be dose related, particularly in the range of 10 to 120 μg/mm^2.

Tabl 1. Summary of the Fibronectin effect on MH

	Nb Rabbits	Nb ulcers Per group	Control group mean MH (mm)	Treatment group mean MH (mm)	p-value
10.3 µg/mm^2	12	48	0.75	0.78	p=0.248
20.6 µg/mm^2	13	52	0.76	0.85	p<0.0001
41.25 µg/mm^2	12	48	0.69	0.77	p<0.0001
82.5 µg/mm^2	13	52	0.67	0.79	p<0.0001
123.75 µg/mm^2	12	48	0.69	0.82	p<0.0001
165 µg/mm^2	12	48	0.66	0.80	p<0.0001

Table 2. Summary of the Fibronectin effect on NGV

	Nb Rabbits	Nb ulcers Per group	Control group mean NGV (mm^3)	Treatment group mean NGV (mm^3)	p-value
10.3 µg/mm^2	12	48	16.34	15.91	p=0.612
20.6 µg/mm^2	13	52	19.66	20.53	p<0.282
41.25 µg/mm^2	12	48	15.52	17.90	p<0.0008
82.5 µg/mm^2	13	52	15.13	18.26	p<0.0001
123.75 µg/mm^2	12	48	16.19	19.75	p<0.0001
165 µg/mm^2	12	48	14.36	15.80	p<0.104

An in *vitro* study, in the deepithelialized skin diffusion cell system, of fibronectin absorption for varying applied doses from 10.3 to 165 µg/mm^2 using the fibronectin-sodium alginate disks of Example 1 was also conducted. The results are shown in Figure 6.

Example 8

Calcium-alginat Disks for D liv ry of High Conc ntrations of Human Plasma Fibronectin

A preferred delivery system of the invention consists of human plasma fibronectin lyophilized in the presence of calcium-alginate to form solid disks, which are to be applied topically to the wound surface to be treated. As opposed to liquid formulations containing fibronectin, the use of calcium-alginate disks helps insure the delivery of known, consistent amounts of fibronectin to a wound surface.

In one embodiment of the invention, human fibronectin is purchased as a cryoprecipitate from a licensed manufacturer (MedImmune, Maryland through DCI Management, New York, NY). The cryoprecipitate extract is first dissolved and clarified. The cryoprecipitate extract is then treated to inactivate lipid enveloped viruses by the solvent/detergent procedure using 0.3% tri (n-butyl) phosphate (TNBP) and 1% Triton X-100 for four hours at room temperature. The process reagents are removed and the fibronectin is purified on a gelatin-Sepharose affinity chromatography. Bound fibronectin is eluted from the column with 1M potassium bromide at pH 5.0. Following the removal of salts, the fibronectin is ~~sterilized~~ concentrated by 100 KD membrane ultrafiltration. sterilized using a 0.22 μm acetate filter and lyophilized. It is then combined with sterile calcium-alginate as described in Example 1. Sterile glass vials are filled aseptically with 5 mL of the fibronectin/calcium alginate mixture under Food and Drug Administration, class 100 good manufacturing procedures.

Stoppers should be inserted partway into the vials and the preparation is dried on a FTS Duralyophilizer lyophilizer. In a preferred embodiment of the invention, the prepared disks, 4.2 cm^2 in diameter, are removed from the vial by forceps, placed on the wound to be treated and wetted with sterile saline (0.9% NaCl). Each dressing holds 80μg of fibronectin per mm^2 of surface area. More than one dressing may be used depending on the size of the skin ulcer or other lesion to be treated and must be trimmed to fit the shape of the

wound. The disks may be stacked to deliver larger doses of fibronectin, such as 160μg per mm^2, 240μg per mm^2, and so forth.

The dressings according to this and previous examples can be prepared using fibronectin from a non-human source. Representative animals include, but are not limited, to horses, dogs and cats.

In one preferred method of treating a human or animal patient, no other ointments, creams or dressings are applied to the wound, ulcer or other lesion while the fibronectin-solid wound dressing of this example is used. The dressing is preferably applied with high compression therapy using a long stretch four-layer bandage.

For example, in a leg lesion, the foot of the patient's bed that is used for sleeping should be raised to form an angle of about 20 degrees. For example, a two-inch block can be placed under the foot of a human patient's bed or two pillows can be placed between the base and the mattress of the bed. If the patient remains seated or lying down for more than 30 minutes after application, he/she should raise his/her legs. Similarly, if the patient remains in a fixed standing position after application, he/she must walk every 30 minutes.

Twice a week, the dressing according to the invention is removed and the wound bed is inspected for evidence of infection. If there are signs of infection, they should be evaluated and treated as necessary by the appropriate health care practitioner. If there is no evidence of infection, then the wound bed is assessed as follows. If at least 50% of the wound bed is pale pink to beefy red (as opposed to gray, yellow, or black indicating necrotic material), then the replacement dressing and compression are applied. If less than 50% of the ulcer bed is pink or red, the wound bed should be debrided, i.e. cleaned of any necrotic material. Debridement should be done with sharp surgical instruments and can be done using local anesthesia.

Example 9

Solubility of Fibron tin

To prepare 10 g of fibronectin carbomer hydrogel, the following ingredients must be added in sequence. First, the pH of 8.8 mL demineralized water, pH 5.0, is adjusted to pH 8.0 to 11.0 by adding 2.95 µg to 2.95 mg NaOH 3M. Demineralized water from different sources may have different starting pH values and the exact amount of NaOH 3M can be adjusted as need to the desired value.

Next, 0.05 to 0.1 grams of lyophilized fibronectin are dissolved in the demineralized water, pH 8.0 to 11.0. In a final step of the procedure, 1 mL of water containing 0.028 g of carbomer and 0.09399705 g to 0.09105 g of NaOH 3M are added to the mixture. Examples of the composition for several fibronectin carbomer hydrogels are shown in Table 2. "FN" stands for lyophilized fibronectin.

Table 3. Composition of fibronectin carbomer hydrogels

Stock solution of carbomer			
3.75 %		2.80 %	
10 g (0.5% FN/ 0.28% Carbomer)	10 g (1.0% FN/ 0.28% Carbomer)	10 g (0.5% FN/ 0.28% Carbomer)	10 g (1.0% FN/ 0.28% Carbomer)
0.75 g Carbomer	0.75 g Carbomer	1.00 g Carbomer	1.00 g Carbomer
0.05 g FN	0.1 g FN	0.05 g FN	0.1 g FN
9.106 g water	9.056 g water	8.856 g water	8.806 g water
0.094 g NaOH 3M	0.094 g NaOH 3M	0.094 g NaOH 3M	0.094 g NaOH 3M

Stock solution of carbomer	
3.75 %	2.80 %

20 g (0.5% FN/ 0.28% Carbomer)	20 g (1.0% FN/ 0.28% Carbomer)	20 g (0.5% FN/ 0.28% Carbomer)	20 g (1.0% FN/ 0.28% Carbomer)
1.50 g Carbomer	1.50 g Carbomer	2.00 g Carbomer	2.00 g Carbomer
0.1 g FN	0.2 g FN	0.1 g FN	0.2 g FN
18.212 g water	18.112 g water	17.712 g water	17.612 g water
0.188 g NaOH 3M	0.188 g NaOH 3M	0.188 g NaOH 3M	0.188 g NaOH 3M

In a particularly preferred embodiment of the fibronectin gel of the invention, the following ingredients must be added in this sequence. First, the pH of demineralized water (8.856 or 8.806 grams) is adjusted to 11.6 with 0.0235 grams of NaOH 3M. 8.856 grams of water is utilized when 0.05 grams of fibronectin will be added; 8.806 grams of water is utilized when 0.1 grams of fibronectin will be added. 0.05 or 0.1 grams of lyophilized fibronectin is next dissolved in the demineralized basic water. In a final step of the procedure, 0.028 grams of carbomer and 0.0705 grams of NaOH 3M are added to the mixture. The 0.028 grams of carbomer comes from 1.0 gram of a 2.8% carbomer stock solution. The concentrated carbomer stock solution may be at 3.75% or 2.80%. The preparation of fibronectin carbomer hydrogels using different concentrated stock solutions is illustrated in Table 2. A preferred grade of carbomer is Carbopol® 974P NF carbomer (BF Goodrich, Cleveland, Ohio). The fibronectin concentration is measured by the well-known micro-Bradford method. The Bradford protein assay is based upon the Bradford dye-binding procedure (Bradford, M. Anal. Biochem. 72, 248, 1976). The protein assay is based on the color change of Coomassie® Brillant Blue G-250 dye, in response to various concentrations of protein.

Table 4. Solubility of fibronectin in n n-buffered water (pH 5.0 – 6.0) compared to the solubility of fibronectin in water with NaOH 0.007 M, pH 11.6

Lyophilized fibronectin (mg)	Measured concentration of fibronectin (micro-Bradford method) (1 h /37°C in water) (mg/mL)	Measured concentration of fibronectin (micro-Bradford method) (12 h /37°C in water) (mg/mL)	Measured concentration of fibronectin by (micro-Bradford method) (24 h /37°C in water) (mg/mL)	Measured concentration of fibronectin by (micro-Bradford method) (1 h /37°C in water + NaOH) (mg/mL)
4	2.1 ± 0.4 (3)*	2.7 ± 0.4 (3)	3.0 ± 0.3 (4)	4.1 ± 0.2 (3)
10	5.7 ± 0.4 (3)	6.3 ± 0.2 (3)	5.7 ± 0.3 (4)	9.0 ± 0.3 (4)
15	7.1 (1)	7.5 (1)	--	--
20	7.3 (1)	8.5 (1)	--	14.1 (1)

*The number in parentheses refers to the number of experiments performed

Table 5. Solubility of fibronectin in water containing varying concentrations of NaOH (10^{-7} M to 10^{-3} M) with corresponding pH (7.0 to 11.0)

Lyophilized fibronectin (mg)	Measured concentration of fibronectin (micro-Bradford method) (1 h /37°C in water + NaOH) (mg/mL)				
10	10^{-7} M NaOH; pH 7.0	10^{-6} M NaOH; pH 8.0	10^{-5} M NaOH; pH 9.0	10^{-4} M NaOH; pH 10.0	10^{-3} M NaOH; pH 11.0
	8.0 (1)*	8.5 (1)	9.2 ± 0.5 (2)	8.7 (1)	9.2 ± 0.3 (5)

*The number in parentheses refers to the number of experiments performed

Example 10

Solv nt/d terg nt-tr at d human homologous plasma fibronectin

The lyophilized fibronectin used in Examples 1-7 and 9 was prepared as described in this example.

First, lots of plasma prepared from different donors are tested for atypical antibodies, hepatitis B and C virus (HBV, HCV), human immunodeficiency virus (HIV), human T-cell lymphotrophic virus (HTLV), cytomegalovirus (CMV) and syphilis.

Second, a viral inactivation solvent/detergent method using tri(n-butyl)phosphate (TNBP) and Triton X-100 is performed. Treatment of plasma products with organic solvent, tri(n-butyl)phosphate (TNBP) and Triton X-100 detergent was shown to inactivate very large quantities of HBV, HCV and HIV (Horowitz et al., 1992, Blood 79 (3):826-31 without affecting labile proteins such as fibronectin.

In a typical preparation, frozen plasma from 5 donors is thawed and treated while stirring for 6 hours with 1% (vol/vol) TNBP, 1% (vol/vol) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride at 24°C. After treatment, soybean oil (20% vol/vol) is added, mixed gently for 30 minutes at ambient temperature, and then removed by using a decantation funnel at 4°C.

Once fibronectin is purified from plasma, for example by the gelatin-Sepharose affinity chromatography procedure described below, the final solution is verified for contamination by TNBP and Triton X-100. TNBP is quantified in a sample of purified fibronectin after hexane extraction by gas chromatography using a 0.25-in by 2 mm ID by 4-ft glass column packed with 10% SP-1000 on a 80/100 mesh Supelcoport (Supelco, Bellafonte, PA). Triton X-100 was assayed by injecting a sample of purified fibronectin to high liquid chromatography (HPLC) on a gel filtration column G2000 SW 7.5 mm ID by 60 cm (Tosohass) coupled with a UV detector set at 230 nm. Fibronectin preparations were found to contain less that 1 ppm of either TNBP or Triton X-100.

Fibronectin was isolated from solvent/detergent-treated human plasma using a gelatin-Sepharose affinity chromatography procedure (Horowitz and Chang, 1989). This method takes advantage of the affinity of fibronectin for gelatin in a procedure that allows isolation of electrophoretically pure fibronectin from human plasma with excellent yields.

In this method, gelatin is covalently coupled to Sepharose CL-4B after CNBr activation. The binding capacity for human plasma fibronectin provided by this system is > 1 mg/mL of gel. The purificaton is performed in a batch procedure with a glass funnel filter holder (Costar Nucleopore, Pleasanton, CA) with a capacity of 375 mL and a filtration area of 10.5 cm^2 at a flow rate of 25 mL/min.

Briefly, the plasma sample is passed twice on a gelatin-Sepharose gel. The matrix is washed with several volumes of 0.15 M Tris-HCl buffer pH 7.5, several volumes of 0.15 M Tris-HCl buffer pH 7.5 + 1 M NaCl and again with 0.15 M Tri-/HCl buffer pH 7.5. Elution is carried out with 1 M KBr in 0.1 M acetate buffer pH 5.0. The resulting solution of fibronectin is then exhaustively dialyzed against deionized and demineralized water, ultrafiltered under nitrogen, lyophilized and frozen at -80°C until used.

The protein concentration is determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). The following diagram summarizes the purification steps.

Fibronectin from non-human animals can be purified using similar methods. The lots of plasma would be screened for atypical antibodies as appropriate for the source organism and known to those skilled in the art.

For example, domestic cat plasma would be screened for feline leukemia virus.

Procedures for purifying fibronectin from human plasma treated with TNBP/Triton X-100

2.5 L of plasma (from 5 donors)

1.0% (v/v) TNBP +
1.0% (v/v) Triton X-100
6 hr/ 24°C

Solvent-Detergent plasma

TNBP extraction with 20% (v/v) vegetable oil

Plasma + 200 mL of gelatin-Sepharose gel

First wash Tris-HCl buffer 0.15 M pH 7.5

Second wash Tris-HCl buffer 0.15 M + 1 M NaCl pH 7.5

Third wash Tris-HCl buffer 0.15 M pH 7.5

Elution sodium acetate buffer 0.1M + 1M KBr pH 5.0

Dialysis against deionized and demineralized water

Concentration by ultrafiltration under nitrogen

Sterile filtration with 0.22 µm acetate filter

Lyophilization

Example 11

Dry h at tr atm nt of pla ma fibron ctin (past urization)

The viral inactivation solvent/detergent method (25°C for 6 h) as described in the previous example has been recognized to be highly effective in the destruction of enveloped-viruses. According to Radosevich (Seminars in Thrombosis and Hemostasis. 24 (2) 157-161,1998), pasteurization has broader virucidal action owing to its additional ability to inactivate non-enveloped viruses. Non-enveloped viruses are usually more resistant to physicochemical treatments than enveloped viruses. Thus, improvements in viral safety for plasma products may be obtained by combining different viral reduction procedures (e.g. solvent-detergent + dry heat treatments).

An additional dry heat treatment on lyophilized solvent-detergent treated-fibronectin at 68°C for 96 hours can be performed before incorporating the fibronectin in the wound formulations of the invention described in the previous examples. For example, in one embodiment of the invention, 50 mg of lyophilized fibronectin prepared according to Example 10 was placed in a 50 mL polypropylene conical tube (Becton Dickinson Labware, Franklin Lakes, NJ) using sterile techniques and sealed. The tube is then submerged in a well-controlled water bath (Exacal Ex-110 water bath Neslab Instruments, Newington, NJ) at 68°C for 96 hours.

When a viral inactivation/removal procedure is implemented, it is important to measure that there is no alteration of protein structure and biological activities. The results of running fibronectin pasteurized as described above and freshly purified fibronectin against molecular weight standards on a standard SDS-PAGE gel showed that the pasteurized fibronectin maintained its structural integrity. The pasteurized fibronectin also demonstrated equivalent activity to freshly purified plasma fibronectin when tested for gelatin binding, cell adhesion promoting activity and chemotactic activity. The freshly purified plasma fibronectin in these tests was prepared according to

the method described in Example 10. Alternatively, the lyophilized fibronectin can be submerged for 72 hours at 80°C. The solid wound healing formulations of Examples 1-5 and Example 8 can also be pasteurized after they have been sealed in the vials.

Example 12

Recombinant fibronectin

Human or veterinary fibronectin produced by recombinant means may be utilized in the solid wound dressings of the invention in place of the plasma fibronectin purified and sterilized according to the methods described in the previous examples. Active fragments of fibronectin or modified fibronectin fragments may be utilized in alternative embodiments of the invention, using methods well known in the art. Such methods include recombinant DNA methods and chemical synthesis methods for production of proteins. For example, recombinant fibronectin polypeptide fragments can be made in bacteria or chemically synthesized. Fibronectin, fibronectin polypeptide fragments or any polypeptide compound used in the invention can be isolated from animal tissue or plasma or produced and isolated from cell culture. They may be produced and isolated from genetically altered animals, such as transgenic animals, to generate more endogenous or exogenous forms of fibronectin. Sequences of fibronectin are known to one skilled in the art, for example, as in Kornblihtt et al., EMBO J. 4:1755-1759 (1985), incorporated herein by reference, or, are available from Genbank, NCBI, NIH, and easily searchable, on the internet at http://www.ncbi.nlm.nih.gov. Publicly available databases are incorporated herein by reference in their entirety.

Representative examples of fibronectin fragments are disclosed in: U.S. Patent No. 5,453,489, entitled "Polypeptide fragments of fibronectin which can modulate extracellular matrix assembly"; U.S.

Patent No. 5,958,874, entitled "Recombinant fibronectin-based extracellular matrix for wound healing"; and U.S. Patent No. 5,922,676, entitled "Methods of inhibiting cancer by using superfibronectin", all of which are incorporated herein by reference in their entirety. Recombinant fibronectin fragments are also available from Takara Shuzo (Otsu, Japan).

Example 13

Wound healing promoters other than fibronectin

The solid wound formulations of the invention can include other wound healing promoter having a composition similar to fibronectin, such as proteins of similar size (extracellular matrix proteins, e.g. throbospondin, laminin, vitronectin, fibrinogen) or smaller size (such as peptides including growth factors, e.g., platelet-derived growth factor). In preferred embodiments of the invention, the appropriate species –specific wound healing promoters are used, i.e., human fibronectin and/or other wound healing promoters for human applications.

Although the present invention has been described in relation to particular embodiments thereof, many other variations, modifications, and uses will become apparent to those skilled in the art. It is therefore understood that numerous variations of the invention can be made which are well within the scope and spirit of this invention as described in the appended claims.

We claim:

1. A solid dressing comprising an effective amount of fibronectin.

2. The solid dressing according to claim 1, which contains at least 0.5 to 1.0% of fibronectin by weight.

3. The solid dressing according to claim 2, wherein the fibronectin comprises plasma fibronectin, recombinant fibronectin, biologically active fragments of plasma fibronectin and biologically active fragments of recombinant fibronectin.

4. The solid dressing according to claim 3, wherein the fibronectin has substantially the same biological activity before and after the fibronectin is incorporated into the dressing.

5. The solid dressing according to claim 3, wherein the fibronectin is human fibronectin.

6. The solid dressing according to claim 3, wherein the fibronectin is fibronectin from a non-human animal.

7. The solid dressing according to claim 3, further comprising an effective amount of a wound healing promoter other than fibronectin.

8. The solid dressing according to claim 7, wherein the wound healing promoter other than fibronectin is selected from the group

consisting of thrombospondin, laminin, vitronectin, fibrinogen, or growth factors.

9. A pharmaceutical delivery system comprising a fibrous dressing containing an effective amount of fibronectin.

10. The pharmaceutical delivery system according to claim 9, wherein the fibrous dressing comprises a plant polysaccharide.

11. The pharmaceutical delivery system according to claim 10, wherein the plant polysaccharide is selected from the group consisting of alginates, carrageenans, and cellulose derivatives.

12. The pharmaceutical delivery system according to claim 9, wherein the fibrous dressing containing an effective amount of fibronectin is solid before contact with an exudating wound and is at least partially a gel after contact with an exudating wound.

13. The pharmaceutical delivery system according to claim 11, comprising a fibronectin-plant polysaccharide dressing wherein the concentration of fibronectin in the fibronectin-plant polysaccharide dressing is about 80μg/mm^2.

14. The pharmaceutical delivery system according to claim 11, comprising a fibronectin-polysaccharide dressing wherein at least 80% of the fibronectin is absorbed in a dermal layer of a deepithelialized skin diffusion cell system after 12 hours.

15. The pharmaceutical delivery system according to claim 9, wherein the fibrous dressing comprises a tissue matrix system.

16. The pharmaceutical delivery system according to claim 9, wherein the fibronectin is human fibronectin.

17. The pharmaceutical delivery system according to claim 9, wherein the fibronectin is fibronectin from a non-human animal.

18. The pharmaceutical delivery system according to claim 9, further comprising an effective amount of a wound healing promoter other than fibronectin.

19. The pharmaceutical delivery system according to claim 18, wherein the wound healing promoter other than fibronectin is selected from the group consisting of thrombospondin, laminin, vitronectin, fibrinogen or growth factors.

20. A method of producing a solid wound dressing according to claim 1 comprising the steps of

a) mixing a dispersion of insoluble fibers and a solution of fibronectin to produce a homogeneous mixture; and

b) freeze-drying the homogeneous mixture of step a to produce a solid wound dressing.

21. The method according to claim 20, wherein the dispersion of insoluble fibers contain some soluble fibers.

22. The method according to claim 20, wherein the insoluble fibers are soluble under some conditions.

23. The method according to claim 20, wherein the solution of fibronectin has a concentration of 10 mg/mL.

24. The method according to claim 20, wherein steps a) and b) are conducted under sterile conditions and comprising the further step of

c) placing and sealing the solid wound dressing of step b) in a sterile container under sterile conditions.

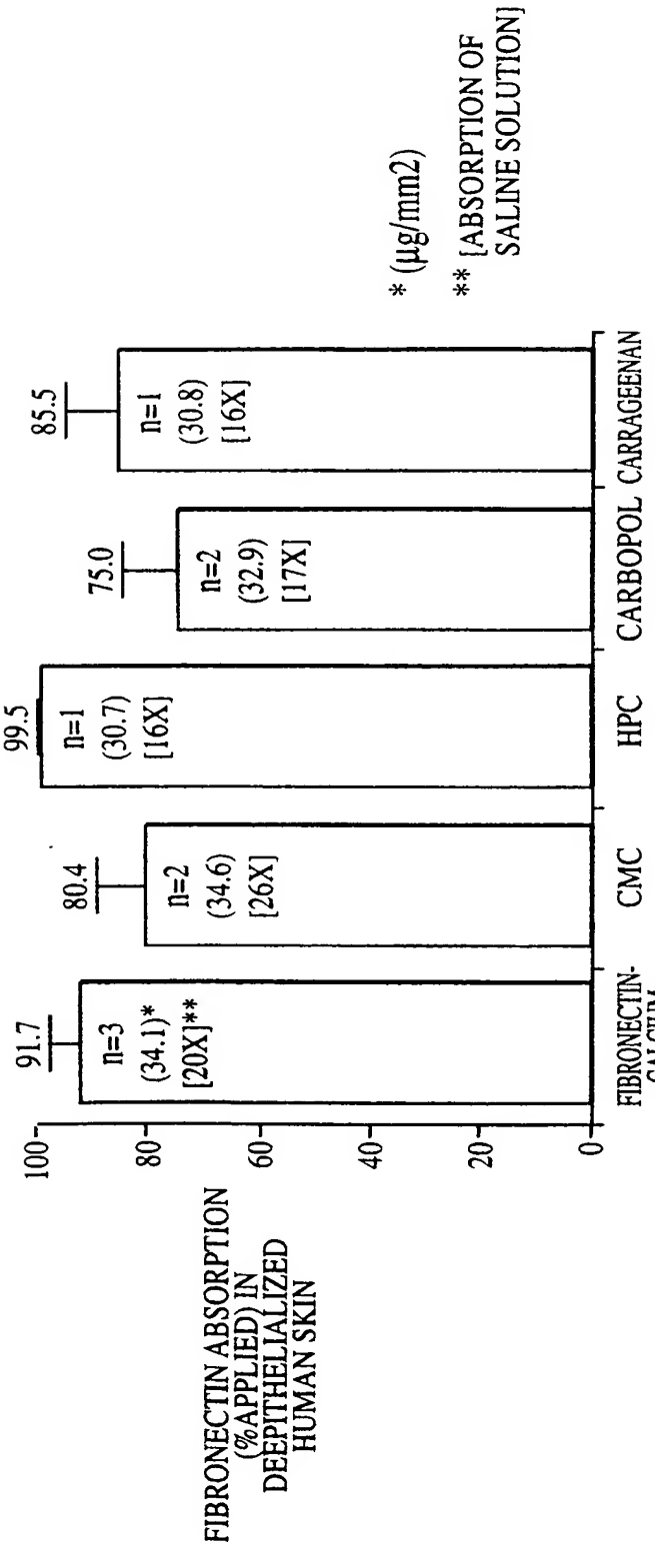

* (µg/mm2)
** [ABSORPTION OF SALINE SOLUTION]
FIBRONECTIN ABSORPTION (%APPLIED) IN DEEPITHELIALIZED HUMAN SKIN
100
80
60
40
20
0
91.7
n=3 (34.1)* [20X]**
80.4
n=2 (34.6) [26X]
99.5
n=1 (30.7) [16X]
75.0
n=2 (32.9) [17X]
85.5
n=1 (30.8) [16X]
FIBRONECTIN-CALCIUM ALGINATE
CMC
HPC
CARBOPOL
CARRAGEENAN
WOUND DRESSINGS

FIG. 1

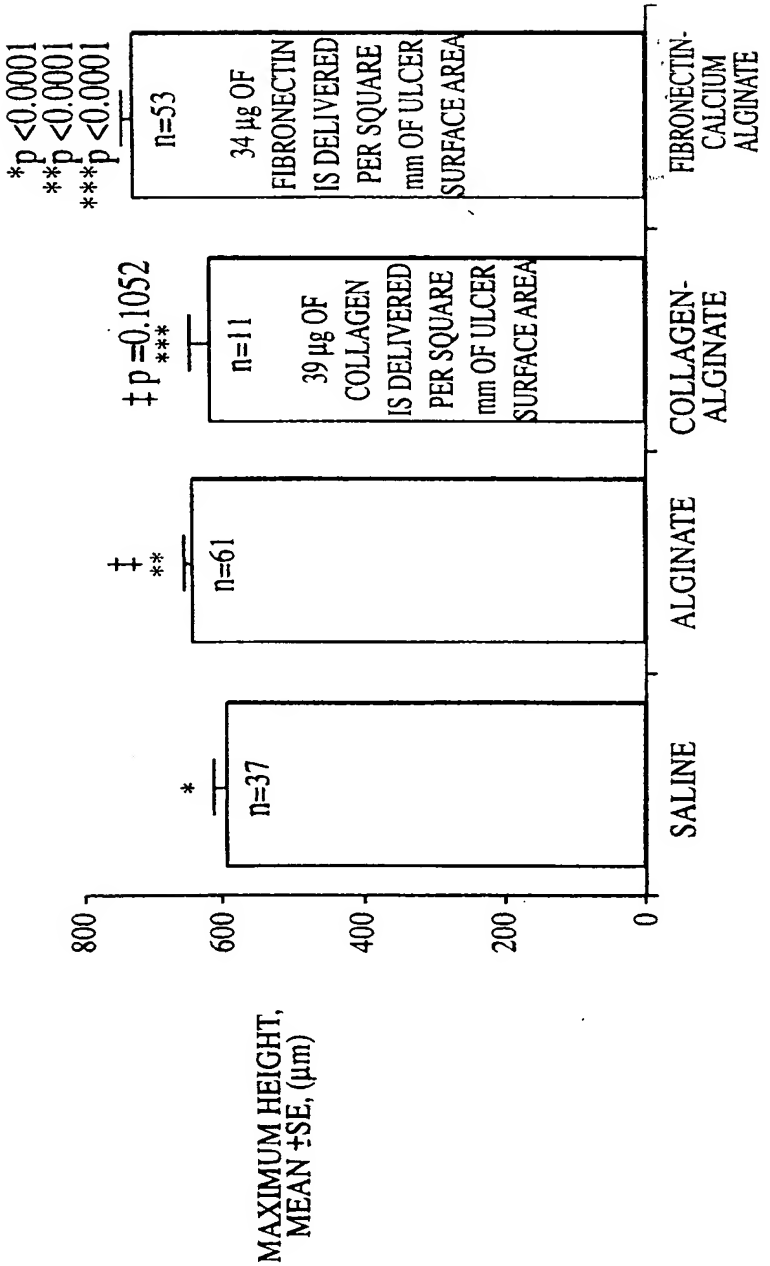

MAXIMUM HEIGHT, MEAN ±SE, (µm)
800
600
400
200
0
n=37
n=61
n=11
n=53
*
‡ **
‡ p =0.1052 ***
*p <0.0001
**p <0.0001
***p <0.0001
34 µg OF FIBRONECTIN IS DELIVERED PER SQUARE mm OF ULCER SURFACE AREA
39 µg OF COLLAGEN IS DELIVERED PER SQUARE mm OF ULCER SURFACE AREA
SALINE
ALGINATE
COLLAGEN-ALGINATE
FIBRONECTIN-CALCIUM ALGINATE

FIG. 2

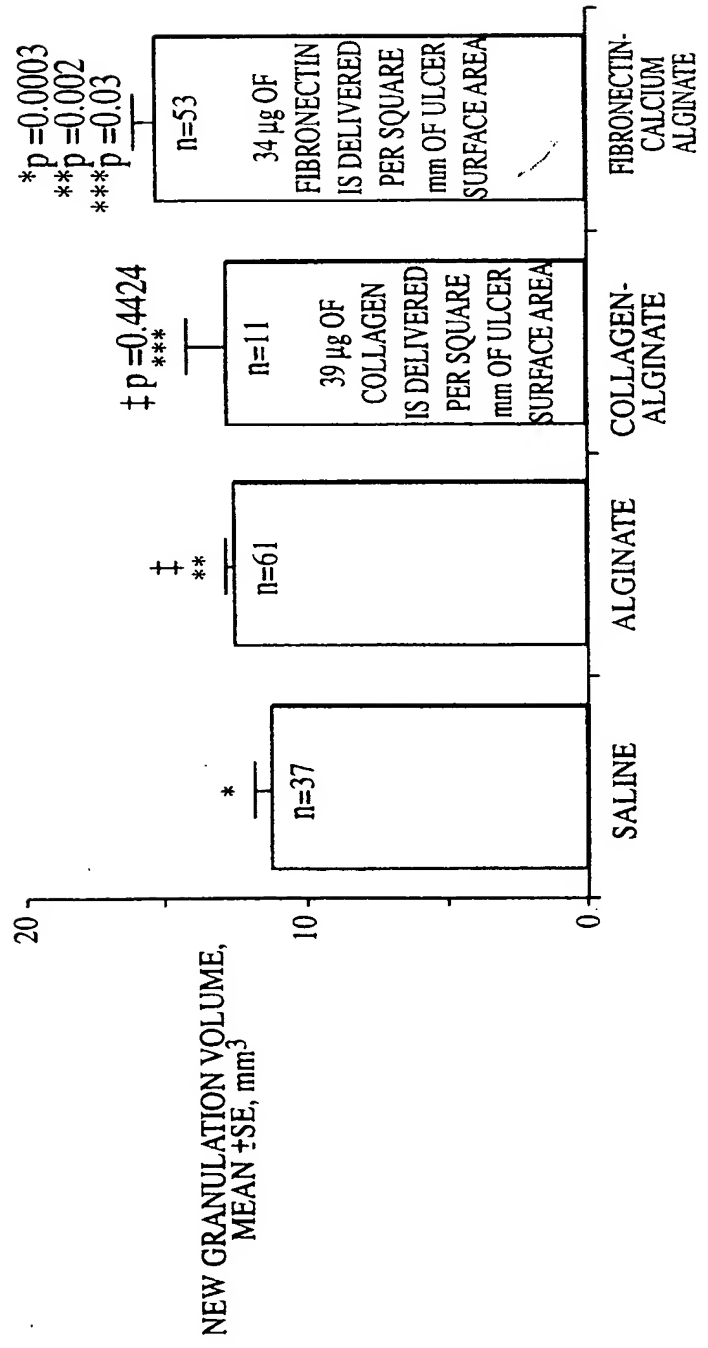

NEW GRANULATION VOLUME, MEAN ±SE, mm3
20
10
0
n=37
n=61
n=11
n=53
*
‡
**
‡ p =0.4424
***
*p =0.0003
**p =0.002
***p =0.03
39 µg OF COLLAGEN IS DELIVERED PER SQUARE mm OF ULCER SURFACE AREA
34 µg OF FIBRONECTIN IS DELIVERED PER SQUARE mm OF ULCER SURFACE AREA
SALINE
ALGINATE
COLLAGEN-ALGINATE
FIBRONECTIN-CALCIUM ALGINATE

FIG. 3

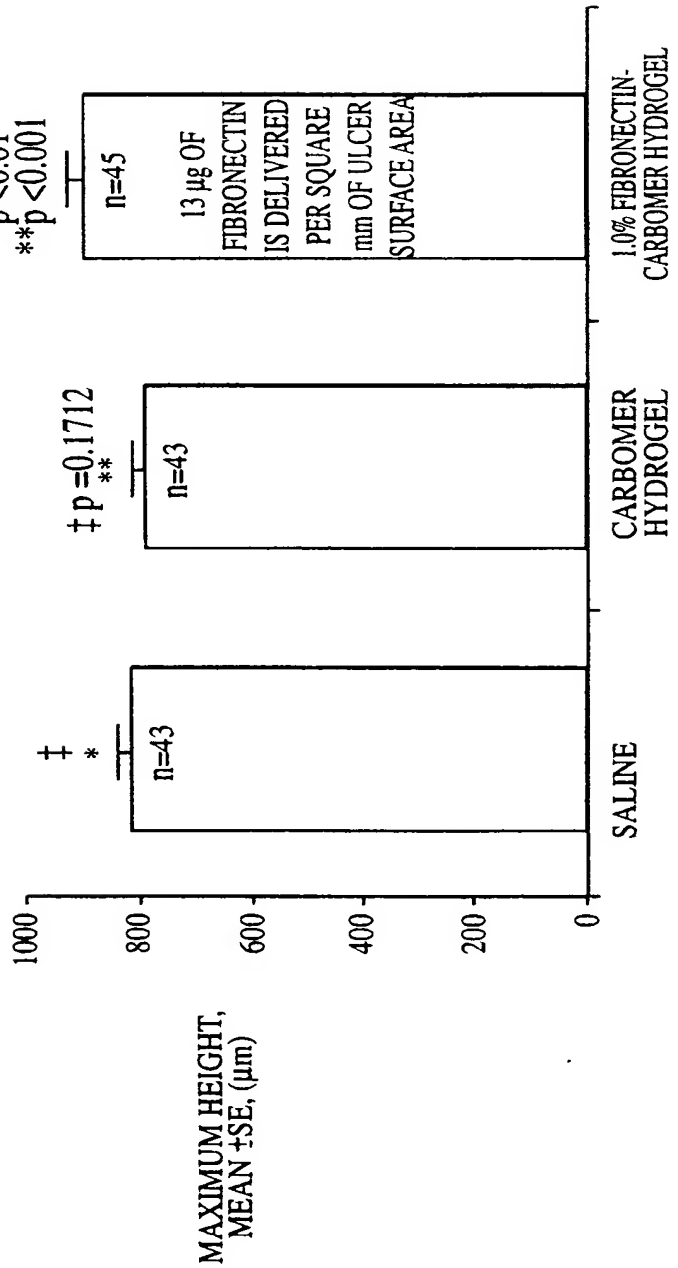

MAXIMUM HEIGHT, MEAN ±SE, (µm)
1000
800
600
400
200
0
‡
*
n=43
‡ p =0.1712
**
n=43
*p <0.01
**p <0.001
n=45
13 µg OF FIBRONECTIN IS DELIVERED PER SQUARE mm OF ULCER SURFACE AREA
SALINE
CARBOMER HYDROGEL
1.0% FIBRONECTIN-CARBOMER HYDROGEL

FIG. 4

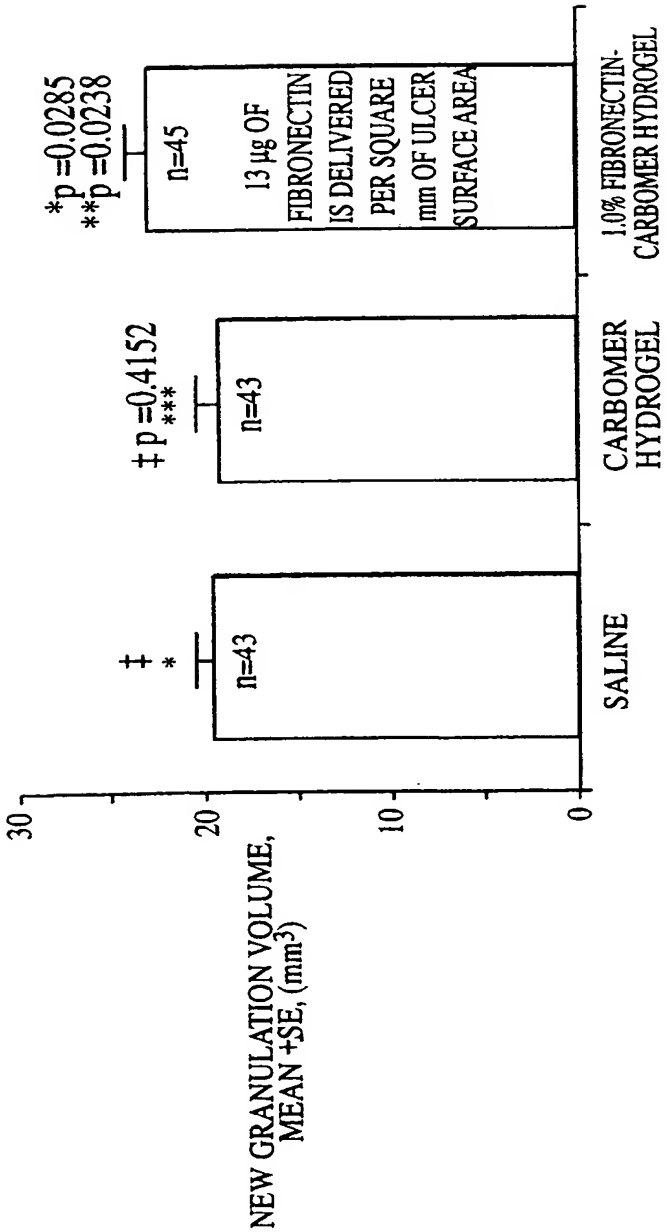

NEW GRANULATION VOLUME, MEAN +SE, (mm3)
30
20
10
0
‡
*
n=43
SALINE
‡p =0.4152
***
n=43
CARBOMER HYDROGEL
*p =0.0285
**p =0.0238
n=45
13 µg OF FIBRONECTIN IS DELIVERED PER SQUARE mm OF ULCER SURFACE AREA
1.0% FIBRONECTIN-CARBOMER HYDROGEL

FIG. 5

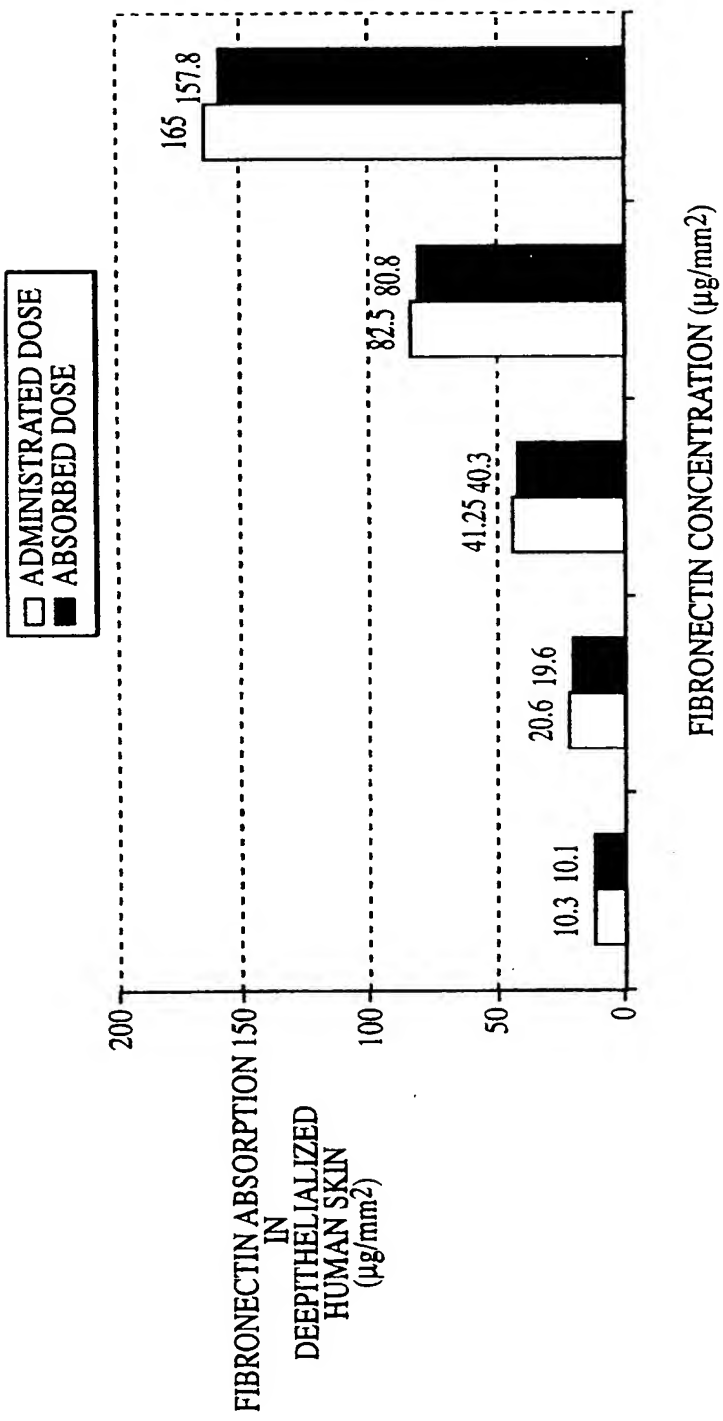

ADMINISTRATED DOSE
ABSORBED DOSE
FIBRONECTIN ABSORPTION IN DEEPITHELIALIZED HUMAN SKIN (µg/mm2)
200
150
100
50
0
165
157.8
82.5
80.8
41.25
40.3
20.6
19.6
10.3
10.1
FIBRONECTIN CONCENTRATION (µg/mm2)

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00953

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L15/28 A61L26/00 C07K14/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 641 483 A (BEAULIEU ANDRE) 24 June 1997 (1997-06-24) claims; examples	1-24
X	EP 0 572 272 A (JOHNSON & JOHNSON MEDICAL) 1 December 1993 (1993-12-01) claims	1-24
X	US 4 784 989 A (HOEOEK MAGNUS ET AL) 15 November 1988 (1988-11-15) column 3, line 1 - line 7; claims	1-24
X	US 5 629 287 A (BLUN GORDON ET AL) 13 May 1997 (1997-05-13) column 3, line 1 - line 7; claims	1-24

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2000	02/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00953

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 610 148 A (BROWN ROBERT) 11 March 1997 (1997-03-11) claims; examples	1-9
X	US 4 973 466 A (REICH CARY) 27 November 1990 (1990-11-27) column 4, line 51 - line 63; claims	1-9
X	US 5 877 149 A (BEAULIEU ANDRE) 2 March 1999 (1999-03-02) cited in the application claims; examples	1-9
A	WO 99 27167 A (UNIV LONDON ;AHMED ZUBAIR (GB); UNDERWOOD SARAH ANNE (GB)) 3 June 1999 (1999-06-03) claims; examples	1-24
A	WO 98 12228 A (WONG WAI HUNG ;UNIV MICHIGAN (US); MOONEY DAVID J (US); ROWLEY JON) 26 March 1998 (1998-03-26) claims	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]al Application No
PCT/CA 00/00953

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5641483 A	24-06-1997	CA 2197244 A	19-12-1996
		WO 9640221 A	19-12-1996
		EP 0784481 A	23-07-1997
		US 5821220 A	13-10-1998
		US 5877149 A	02-03-1999
EP 0572272 A	01-12-1993	AT 183103 T	15-08-1999
		AU 3878693 A	02-12-1993
		BR 9302089 A	07-12-1993
		CA 2097268 A	30-11-1993
US 4784989 A	15-11-1988	SE 446688 B	06-10-1986
		AT 35380 T	15-07-1988
		DE 3377198 D	04-08-1988
		DK 234784 A,B,	11-05-1984
		EP 0119233 A	26-09-1984
		FI 841936 A	14-05-1984
		JP 5049302 B	23-07-1993
		JP 59501745 T	18-10-1984
		NO 841901 A	11-05-1984
		NO 164639 B	23-07-1990
		SE 8205244 A	15-03-1984
		WO 8401108 A	29-03-1984
US 5629287 A	13-05-1997	AT 191855 T	15-05-2000
		AU 660447 B	29-06-1995
		AU 1166692 A	27-08-1992
		CH 684783 A	30-12-1994
		DE 69230932 D	25-05-2000
		DE 69230932 T	14-12-2000
		EP 0567528 A	03-11-1993
		ES 2085199 A	16-05-1996
		WO 9212739 A	06-08-1992
		JP 6504546 T	26-05-1994
US 5610148 A	11-03-1997	AU 662314 B	31-08-1995
		AU 1172292 A	27-08-1992
		CH 684271 A	15-08-1994
		EP 0567508 A	03-11-1993
		ES 2085198 A	16-05-1996
		WO 9213003 A	06-08-1992
		JP 6504926 T	09-06-1994
US 4973466 A	27-11-1990	US 5124155 A	23-06-1992
US 5877149 A	02-03-1999	US 5641483 A	24-06-1997
		CA 2197244 A	19-12-1996
		WO 9640221 A	19-12-1996
		EP 0784481 A	23-07-1997
		US 5821220 A	13-10-1998
WO 9927167 A	03-06-1999	AU 1342299 A	15-06-1999
		EP 1034326 A	13-09-2000
WO 9812228 A	26-03-1998	AU 4493097 A	14-04-1998
		EP 0927196 A	07-07-1999

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PCT 15758-15		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00953	21/08/2000	20/08/1999
Applicant		
BEAULIEU, Andre et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 3

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00953

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L15/28 A61L26/00 C07K14/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 641 483 A (BEAULIEU ANDRE) 24 June 1997 (1997-06-24) claims; examples	1-24
X	EP 0 572 272 A (JOHNSON & JOHNSON MEDICAL) 1 December 1993 (1993-12-01) claims	1-24
X	US 4 784 989 A (HOEOEK MAGNUS ET AL) 15 November 1988 (1988-11-15) column 3, line 1 - line 7; claims	1-24
X	US 5 629 287 A (BLUN GORDON ET AL) 13 May 1997 (1997-05-13) column 3, line 1 - line 7; claims	1-24

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2000	02/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 610 148 A (BROWN ROBERT) 11 March 1997 (1997-03-11) claims; examples	1-9
X	US 4 973 466 A (REICH CARY) 27 November 1990 (1990-11-27) column 4, line 51 - line 63; claims	1-9
X	US 5 877 149 A (BEAULIEU ANDRE) 2 March 1999 (1999-03-02) cited in the application claims; examples	1-9
A	WO 99 27167 A (UNIV LONDON ;AHMED ZUBAIR (GB); UNDERWOOD SARAH ANNE (GB)) 3 June 1999 (1999-06-03) claims; examples	1-24
A	WO 98 12228 A (WONG WAI HUNG ;UNIV MICHIGAN (US); MOONEY DAVID J (US); ROWLEY JON) 26 March 1998 (1998-03-26) claims	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 00/00953

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5641483 A	24-06-1997	CA 2197244 A	19-12-1996
		WO 9640221 A	19-12-1996
		EP 0784481 A	23-07-1997
		US 5821220 A	13-10-1998
		US 5877149 A	02-03-1999
EP 0572272 A	01-12-1993	AT 183103 T	15-08-1999
		AU 3878693 A	02-12-1993
		BR 9302089 A	07-12-1993
		CA 2097268 A	30-11-1993
US 4784989 A	15-11-1988	SE 446688 B	06-10-1986
		AT 35380 T	15-07-1988
		DE 3377198 D	04-08-1988
		DK 234784 A,B,	11-05-1984
		EP 0119233 A	26-09-1984
		FI 841936 A	14-05-1984
		JP 5049302 B	23-07-1993
		JP 59501745 T	18-10-1984
		NO 841901 A	11-05-1984
		NO 164639 B	23-07-1990
		SE 8205244 A	15-03-1984
		WO 8401108 A	29-03-1984
US 5629287 A	13-05-1997	AT 191855 T	15-05-2000
		AU 660447 B	29-06-1995
		AU 1166692 A	27-08-1992
		CH 684783 A	30-12-1994
		DE 69230932 D	25-05-2000
		DE 69230932 T	14-12-2000
		EP 0567528 A	03-11-1993
		ES 2085199 A	16-05-1996
		WO 9212739 A	06-08-1992
		JP 6504546 T	26-05-1994
US 5610148 A	11-03-1997	AU 662314 B	31-08-1995
		AU 1172292 A	27-08-1992
		CH 684271 A	15-08-1994
		EP 0567508 A	03-11-1993
		ES 2085198 A	16-05-1996
		WO 9213003 A	06-08-1992
		JP 6504926 T	09-06-1994
US 4973466 A	27-11-1990	US 5124155 A	23-06-1992
US 5877149 A	02-03-1999	US 5641483 A	24-06-1997
		CA 2197244 A	19-12-1996
		WO 9640221 A	19-12-1996
		EP 0784481 A	23-07-1997
		US 5821220 A	13-10-1998
WO 9927167 A	03-06-1999	AU 1342299 A	15-06-1999
		EP 1034326 A	13-09-2000
WO 9812228 A	26-03-1998	AU 4493097 A	14-04-1998
		EP 0927196 A	07-07-1999

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WRAY, Robert, William
R. William Wray & Associates
310-151 Slater Street
Ottawa, Ontario K1P 5H3
CANADA

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	11.12.2001
Applicant's or agent's file reference PCT 15758-15	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00953	21/08/2000	20/08/1999

Applicant
BEAULIEU, Andre et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Senkel, H Tel.+49 89 2399-8071

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PCT 15758-15		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00953	21/08/2000	20/08/1999
International Patent Classification (IPC) or national classification and IPC A61L15/28		
Applicant BEAULIEU, Andre et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/03/2001	11.12.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Skjöldebrand, C Telephone No. +49 89 2399 8467

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00953

I. Basis of the r port

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-33 as originally filed

Claims, No.:

1-24 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	20-24
	No:	Claims	1-19
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-24
Industrial applicability (IA)	Yes:	Claims	1-24
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re It m V

Reasoned statement under Article 35(2) with regard to novelty, inventive step r industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: US-A-5 641 483 (BEAULIEU ANDRE) 24 June 1997 (1997-06-24)

D2: EP-A-0 572 272 (JOHNSON & JOHNSON MEDICAL) 1 December 1993 (1993-12-01)

D3: US-A-4 784 989 (HOEOEK MAGNUS ET AL) 15 November 1988 (1988-11-15)

D4: US-A-5 629 287 (BLUN GORDON ET AL) 13 May 1997 (1997-05-13)

D5: US-A-5 610 148 (BROWN ROBERT) 11 March 1997 (1997-03-11)

D6: US-A-4 973 466 (REICH CARY) 27 November 1990 (1990-11-27)

D7: US-A-5 877 149 (BEAULIEU ANDRE) 2 March 1999 (1999-03-02) cited in the application

Novelty - Article 33(2) PCT

Document D1 relates to topical dosage forms containing human plasma fibronectin for use in promoting wound healing in humans. Aqueous gel formulations containing 0.05 to about 0.5% by weight of human plasma fibronectin or fibronectin obtained from recombinant-DNA technology, and a water soluble, pharmaceutically acceptable polymer are disclosed. Alternative wound healing promoters are mentioned, for example thrombospondin, laminin, vitronectin, fibrinogen or peptides including growth factors. Carbomer (Carbopol) and cellulose derivatives, including CMC (sodium carboxymethylcellulose) and HPC (hydroxypropylcellulose), may be comprised in the formulations (see claims and examples).

D2 discloses preparations for use as bone sealants. Comprised in said compositions

are fibrous proteins, including fibronectin, fibrin and laminin, a tackifying agent including cellulose derivatives and a mucopolysaccharide, e.g. alginate and gum. The resulting composition is cast into tablets (example 1).

D3 discloses methods of treating wounds infected by microorganisms. Wound healing promoters such as fibronectin, fibrinogen, laminin are comprised and may be delivered using dressing containing a fibrous material, e.g. cellulose derivatives and alginate (col. 3, lines 1-7 and claims).

D4 discloses depot formulations which provide prolonged release of wound healing promoters into wounds in humans and animals. Preferably the materials are used as, or as part of, a wound dressing (col. 5, lines 46-47). The fibrils of the fibronectin or fragment thereof are in the form of a porous macroscopically oriented material. Other therapeutic agents may be included, such as growth factors and fibrinogen.

D5 discloses a wound dressing comprising a porous macroscopically unidirectionally oriented cell adhesion protein selected from fibronectin, vitronectin and von Willebrand protein, in which fibrils of the cell adhesion protein are macroscopically unidirectionally oriented (see claims and examples)

D6 relates to wound-healing dressings and to methods for enhancing the quality of wound healing. A method for preparing such dressings is disclosed which comprises reacting a solution containing fibronectin or a biologically active fragment or analog thereof with an agent which changes the pH or osmolarity of the solution thereby forming a flocculent; recovering the flocculent from the solution; forming and drying the flocculent to produce a solid, water-swellable and substantially water-insoluble wound-healing dressing (claims). The gel may include other wound healing promoters, including laminin, vitronectin and fibrinogen (col. 4, line 51-63).

D7 also relates to aqueous gel formulations containing fibronectin and their use for the delivery of an effective wound healing amount of fibronectin to a wound site. Alternatives to fibronectin are thrombospondin, laminin, vitronectin, fibrinogen and growth factors. Cellulose derivatives, such as CMC and HPC and Carbopol may be added. The formulations can be used to coat fibres of an absorbent gauze dressing to form a wound healing bandage which may then be placed on a wound (col. 6, line 56-

58).

The disclosure of documents D1-D7 appears to destroy novelty for product claims 1-19.

Inventive Step - Article 33(3) PCT

Could the Applicant show that any subject-matter of product claims 1-19 was to be novel, it doesn't appear to involve any inventive step. Solid dressings used to deliver the fibronectin based on calcium alginate and carrageenan seems to be obvious alternatives to cellulose derivatives.

Freeze drying of the product to solidify it and packaging it under sterile conditions are steps obvious to the skilled man. Hence, the subject-matter of method claims 20-24 appears not to involve an inventive step.

Re Item VII

Certain defects in the international application

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D2-D6 is not discussed in the description, nor are these documents identified therein.

Re Item VIII

Certain observations on the international application

The following terms and definitions are considered unclear in that they don't specify any technical feature or in that a technical feature is defined by referring to a desirable property (Art. 6 PCT).

An "effective amount of fibronectin" (claims 1, 9)

"wherein the fibronectin has substantially the same biological activity before and after the fibronectin is incorporated into the dressing" (claim 4)

"is solid before contact with an exudating wound and is at least partially a gel after contact with an exudating wound" (claim 12)